



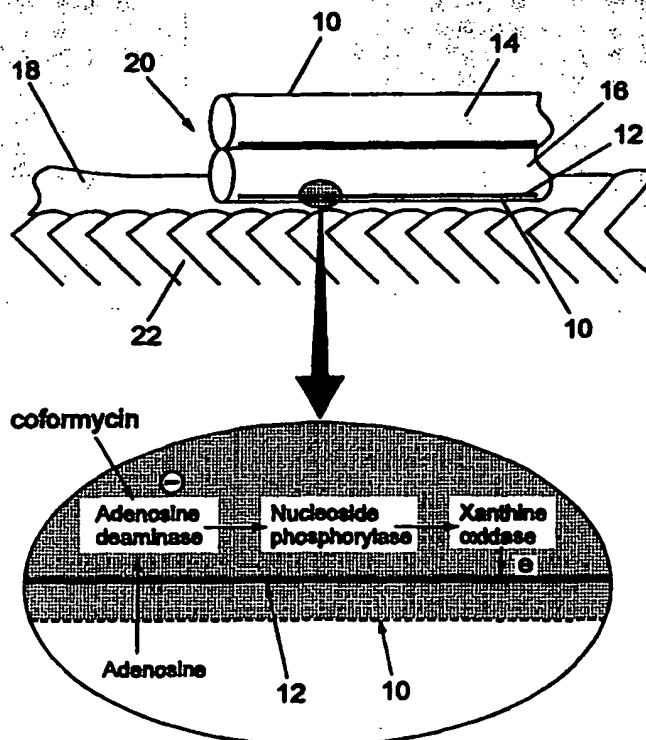
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>6</sup> : <b>C12Q 1/00, G01N 27/327, C12Q 1/34, 1/48, 1/26</b></p>	<p><b>A1</b></p>	<p>(11) International Publication Number: <b>WO 99/07877</b></p> <p>(43) International Publication Date: 18 February 1999 (18.02.99)</p>
<p>(21) International Application Number: <b>PCT/GB98/02239</b></p> <p>(22) International Filing Date: <b>5 August 1998 (05.08.98)</b></p> <p>(30) Priority Data:              9716453.7                      5 August 1997 (05.08.97)      GB              9725231.6                      29 November 1997 (29.11.97)      GB</p> <p>(71) Applicant (for all designated States except US): <b>THE UNIVERSITY COURT OF THE UNIVERSITY OF ST. ANDREWS [GB/GB]; College Gate, North Street, St. Andrews KY16 9AJ (GB).</b></p> <p>(72) Inventor; and          (75) Inventor/Applicant (for US only): <b>DALE, Nicholas, Egerton [GB/GB]; 38 Markegate S, Crail KY10 3TL (GB).</b></p> <p>(74) Agent: <b>MURGITROYD &amp; COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).</b></p>	<p>(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b>  <i>With international search report.</i></p>	

(54) Title: **BIOSENSOR FOR DETECTING ADENOSINE**

## (57) Abstract

A biosensor to detect adenosine which comprises the enzymes adenosine deaminase, nucleoside phosphorylase and xanthine oxidase, or functional equivalents thereof, immobilized on a support and means to detect hydrogen peroxide like an electrolytic cell. Detection of adenosine level can be particularly useful, for example, in the treatment of narcolepsy, heart surgery and to improve the effect of some medications.



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1  
2       BIOSENSOR FOR DETECTING ADENOSINE

3  
4       The present invention relates to a biosensor and assay  
5       for detecting adenosine.

6  
7       Adenosine is an important and near universal  
8       neuromodulator in the peripheral and central nervous  
9       systems. In the brain adenosine functions to protect  
10       cells against ischaemic damage. Additionally,  
11       adenosine has been implicated in the regulation of pain  
12       pathways, the control of REM sleep, regulation of  
13       spinal motor patterns and synaptic plasticity  
14       underlying memory. Peripherally adenosine is  
15       powerfully regulated in blood plasma and may be  
16       involved in regulation of blood pressure and other  
17       autonomic functions.

18  
19       To facilitate study of adenosine various sensitive  
20       methods have been developed for measuring adenosine  
21       levels. However, such methods ultimately require  
22       running a sample through a High Performance Liquid  
23       Chromatography (HPLC) machine. Consequently, current  
24       methods suffer from the disadvantages due to the  
25       absolute requirement for such expensive machinery,

1 including lack of portability, the necessity of a  
2 skilled operator and the time required to perform a  
3 measurement. Methodologies reliant upon HPLC  
4 techniques also exhibit limited time resolution.

5  
6 There thus exists a need to develop techniques enabling  
7 rapid time resolution of adenosine content in a sample.  
8 Desirably, such techniques would involve only portable,  
9 inexpensive equipment capable of providing rapid  
10 measurements by relatively unskilled operators.

11  
12 Monitoring of adenosine presence and/or content may be  
13 of particular utility in the following situations:

14  
15 Narcolepsy: is a disorder of REM sleep where the  
16 affected individual will experience irresistible  
17 sleep attacks of 5 to 30 minutes throughout the  
18 day. The incidence of narcolepsy is 0.04 to 0.09%  
19 of the population and very often its sufferers go  
20 undiagnosed and suffer unwarranted social stigma  
21 for apparent laziness. Since adenosine may be  
22 involved in turning on REM sleep, it is possible  
23 that inadequate regulation of adenosine release  
24 could be a contributing factor. This in turn  
25 suggests that measurement of adenosine levels in  
26 narcoleptics could have diagnostic value.

27  
28 Effective Medication: many drugs are often only  
29 effective if their levels in plasma (and CSF) are  
30 kept at therapeutic levels. Elevation of  
31 adenosine may be desirable to protect neural  
32 damage following stroke, and a suitable  
33 measurement method would allow a drug treatment  
34 regime to be tailored to achieve the correct  
35 levels of adenosine.  
36

1        Heart Surgery: Adenosine protects the heart during  
2        transient oxygen deprivation, by increasing the  
3        supply of blood to the heart, and reducing the  
4        work performed by the heart. Clinically,  
5        adenosine and drugs which target either adenosine  
6        degradation or reuptake are used to treat a  
7        variety of conditions. Abnormal heart rhythms can  
8        be terminated by transient application of  
9        adenosine. During heart surgery, the blood supply  
10       to the heart muscle is stopped. When the surgery  
11       is complete, reperfusion of the heart with blood  
12       causes damage to the muscle which can be greatly  
13       reduced by treatment with adenosine. However the  
14       problem with using adenosine as a treatment is  
15       that its actions depend upon the mode and locus of  
16       application as well the dose. To compound these  
17       problems even further, adenosine has a very short  
18       half life in blood (seconds to minutes)..  
19       Furthermore, if the patients are already on drugs  
20       which modify adenosine uptake or degradation there  
21       is even further uncertainty over dosage.  
22  
23       The ability to determine adenosine levels in the  
24       blood rapidly on-site (e.g. in an operating  
25       theatre during surgery or in an Outpatient  
26       Department) would remove uncertainty about dosage  
27       and would allow optimal treatment with adenosine  
28       and thus greatly improve the efficiency of  
29       treatment.  
30  
31       EP-A-0184909 to Alberly describes an enzymically based  
32       probe which may include the enzyme xanthine oxidase.  
33       However, there is no description of a probe capable of  
34       monitoring or detecting adenosine.  
35  
36       The present invention provides a bio-sensor comprising

1 the enzymes adenosine deaminase, nucleoside  
2 phosphorylase and xanthine oxidase (or functional  
3 equivalents thereof) and means to detect hydrogen  
4 peroxide. Desirably, the enzymes are in an aqueous  
5 environment, for example, are in aqueous solution.

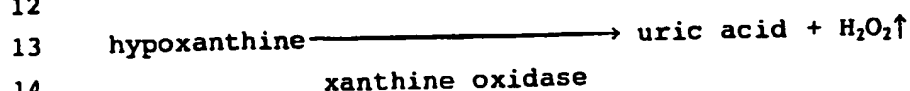
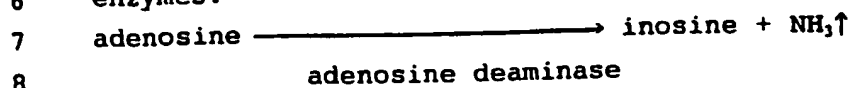
6  
7 Generally, the enzymes will be entrapped by a suitable  
8 means, for example, a semi-porous membrane, although  
9 any means which enables the enzymes to interact with  
10 substrates in an aqueous phase whilst retaining the  
11 enzymes in a particular locality will be suitable.  
12 Suitable semi-porous membranes include semi-permeable  
13 glass membranes, for example of the type made by  
14 Sycopel International. One convenient form of hydrogen  
15 peroxide detecting means to be used in the biosensor is  
16 an electrolytic cell. It may comprise a single or a  
17 dual-barrelled probe each consisting of a 230µm  
18 diameter semipermeable cylindrical glass membrane, a  
19 working electrode (eg. Pt electrode), a counter  
20 electrode (eg. Ag electrode) and a reference electrode  
21 (eg. Ag-AgCl electrode). The dual-barrelled probes  
22 could be used as a quasi-differential device, in that  
23 enzymes can be loaded into only one barrel and the  
24 difference signal between the two barrels measured.

25  
26 When placed into a sample containing adenosine the  
27 three enzymes will act in series to convert adenosine  
28 to uric acid with the evolution of hydrogen peroxide as  
29 a by-product. The rate of production of hydrogen  
30 peroxide is therefore proportional to the concentration  
31 of adenosine. The hydrogen peroxide can then be  
32 detected, for example, by using a platinum electrode.  
33 Our experiments have shown that adenosine  
34 concentrations as low as 10nM can be detected in this  
35 way. One advantage of the bio-sensor of the present  
36 invention is that it enables adenosine concentration to

1 be monitored in real time.

2

3 The sequential action of the enzymes involved in the  
4 present invention can be described by the following  
5 equations which illustrate the order of action of the  
6 enzymes:



15  
16 The relative concentrations of neighbouring enzymes (ie  
17 adenosine deaminase: nucleoside phosphorylase and  
18 nucleoside phosphorylase: xanthine oxidase) will affect  
19 the efficiency of the bio-sensor since product  
20 inhibition may cause a decay in the response observed.  
21 Ratios of adenosine deaminase: nucleoside phosphorylase  
22 of from 1:100 to 1:1 (especially 1:10 to 1:1) and  
23 ratios of nucleoside phosphorylase: xanthine oxidase of  
24 from 1:100 to 1:10 (especially 1:50 to 1:10) are  
25 satisfactory. In general a relative increase in the  
26 concentrations of enzymes used (in the order adenosine  
27 deaminase: nucleoside phosphorylase: xanthine oxidase)  
28 is required. Examples of suitable such ratios are  
29 1:200:500 which has an efficiency of approximately 50%  
30 and 1:2:100 which has an efficiency of approximately  
31 80%. A ratio of adenosine deaminase: nucleoside  
32 phosphorylase: xanthine oxidase in the range 1:1:50 to  
33 1:5:200 is preferred and a ratio of approximately  
34 1:2:100 is especially preferred.

35

36 In a further aspect, the present invention provides a

1 method of detecting adenosine in a sample, said method  
2 comprising exposing the sample to the enzymes adenosine  
3 deaminase, nucleoside phosphorylase and xanthine  
4 oxidase (or functional equivalents thereof) such that  
5 the enzymes can act sequentially on the sample, and  
6 measuring the production of hydrogen peroxide. The  
7 amount of hydrogen peroxide provided is directly  
8 proportional to the amount of adenosine in the sample.  
9 If required, the evolution of hydrogen peroxide can be  
10 measured over time to enable adenosine content to be  
11 monitored, for example, in real time.

12  
13 In yet a further aspect, the present invention provides  
14 a method of diagnosis and treatment of pathological  
15 conditions that result from faulty regulation of  
16 adenosine, said method comprising detecting the levels  
17 of adenosine in a patient in the manner described  
18 above. For example the method of the invention can be  
19 used in diagnosing sleep disorders (such as  
20 narcolepsy).

21  
22 In still a further aspect, the present invention  
23 provides a method of monitoring drug requirements in a  
24 patient, wherein said drug affects the *in vivo* levels  
25 of free adenosine in a body fluid or an organ of said  
26 patient, said method comprising detecting the level of  
27 adenosine in said fluid or organ in the manner  
28 described above.

29  
30 In a yet further aspect, the present invention provides  
31 a method of monitoring adenosine levels in a patient  
32 before, during and/or after surgery, wherein the  
33 adenosine levels are detected in the manner described  
34 above. A particular example is the monitoring of  
35 adenosine levels in the blood supply to the heart at  
36 least during part of a cardiac surgical procedure in



1 order to ensure that, if necessary, adenosine levels  
2 are boosted to the levels required to combat damage to  
3 the cardiac muscle following reperfusion of the heart.  
4 Conveniently the adenosine levels are monitored  
5 continuously or intermittently at appropriate time  
6 intervals by use of the bio-sensor of the present  
7 invention.

8  
9 In certain samples there may be electro-active species  
10 which are also present. These electro-active species  
11 could interact non-specifically with the platinum  
12 electrode of the bio-sensor and influence the accuracy  
13 of the result obtained. Such non-specific interactions  
14 should desirably be filtered out from the final reading  
15 in order to obtain accurate correlation of hydrogen  
16 peroxide production with adenosine content.

17  
18 In a modification of the method described above, it is  
19 envisaged that the biosensor is placed into the sample  
20 of interest and a stable reading obtained, this reading  
21 being the sum of the interaction at the electrode due  
22 to evolution of hydrogen peroxide and also the activity  
23 arising from any non-specific electro-active species  
24 present. In the modification a specific inhibitor to  
25 adenosine deaminase is then introduced. The inhibitor  
26 would block the first reaction of the series,  
27 preventing hydrogen peroxide production. Consequently,  
28 the portion of the final signal due to adenosine  
29 presence obtained after inhibitor introduction would  
30 cease. In other words, the reduced signal will be due  
31 solely to the presence of electro-active species  
32 interacting non-specifically with the platinum  
33 electrode. This reduced reading would then be  
34 subtracted from the initial reading to produce the  
35 signal due only to adenosine presence. Suitable  
36 inhibitors for adenosine deaminase include EHNA

1 (erythro-9-(2-hydroxy-3-nonyl)adenine) and coformycin.  
2 Further information regarding adenosine uptake systems  
3 may be obtained by using a blocker of adenosine uptake,  
4 for example NBTG (S-(4-nitrobenzyl)-6-thioguanosine).

5  
6 The platinum electrode used for hydrogen peroxide  
7 detection in the present invention may be connected to  
8 a potentiostat which holds the voltage of the electrode  
9 constant at +650mV. Suitable equipment is manufactured  
10 by Sycopel. It is possible for a reference electrode  
11 to be included in the bio-sensor, although this is not  
12 essential. A suitable reference electrode could  
13 consist of the last two enzymes placed into a buffer  
14 solution.

15  
16 In a further aspect, the present invention provides the  
17 sequential use of the enzymes adenosine deaminase,  
18 nucleoside phosphorylase and xanthine oxidase in a bio-  
19 sensor. Generally, the bio-sensor will be adapted to  
20 monitor adenosine and will be used in conjunction with  
21 a means for detecting hydrogen peroxide.

22  
23 The technique described above has been used to measure  
24 the release of adenosine from *Xenopus* embryo spinal  
25 cord during swimming. Adenosine is produced from the  
26 ventral part of the spinal cord and builds up slowly  
27 during swimming episodes before decaying back to  
28 baseline levels once the activity has finished. Our  
29 experiments provide the first demonstration that  
30 adenosine is released by the spinal cord during motor  
31 activity. This is also the first time that adenosine  
32 production has been monitored in real time during  
33 neural activity.

34  
35 The invention is further illustrated by the following,  
36 non-limiting, examples and drawings:

# BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. shows the detection of adenosine concentration *in vitro* by enzyme microprobe obtained with a biosensor of the invention.

Figure 2. shows the detection of adenosine-release during swimming activity in a *Xenopus* embryo.

Figure 3. shows a schematic representation of the way a biosensor of the invention is working and the biochemical principle behind enzymatic-electrochemical detection of adenosine.

Figure 4. shows an *in vitro* calibration and characterization of an adenosine biosensor of the invention.

Figure 5. shows how a biosensor probe of the invention can detect adenosine released from the spinal cord during fictive locomotion.

Figure 6. shows how blockers of adenosine uptake greatly enhanced the release of adenosine from the spinal cord.

## Example 1

### Loading biosensor probes with enzyme solutions

0.001U of adenosine deaminase (type VII, SIGMA), 0.2U of nucleoside phosphorylase (from calf spleen, SIGMA), and 5U of xanthine oxidase (from micro-organism, SIGMA) were dissolved in 40 $\mu$ l of a saline consisting of 115mM NaCl, 1mM NaP<sub>i</sub>, 10mM HEPES, pH 7.4. 10 $\mu$ l of this solution was then introduced into a biosensor probe (SYCOPEL) at a flow rate of 60 $\mu$ l/hour.

### In vitro calibration

A Biosensor Driver (SYCOPEL) was used to hold the probe at +650mV and recorded any current signals generated.

1 When dual probes were used in a differential mode (with  
2 enzymes being present only in one barrel) two Biosensor  
3 Drivers were used (one for each probe) and the  
4 difference signal between the two was obtained by a  
5 differential amplifier. Probes were calibrated by  
6 placing them in a continuously stirred bath with a  
7 volume of 7ml. Concentrated aliquots of adenosine were  
8 successively added to give the desired bath  
9 concentration of adenosine. Greatest stability was  
10 achieved when the probe and bath were shielded from all  
11 air currents. The results are given in Figure 1.

## 12 13 Example 2

### 14 15 Recording adenosine release during swimming in *Xenopus* 16 embryos

17  
18 Stage 37/38 *Xenopus* embryos were paralysed with  $\alpha$ -  
19 bungarotoxin and prepared for physiological recordings  
20 using well established techniques (eg Dale, N. 1995  
21 "Experimentally derived model for the locomotor pattern  
22 generator in the *Xenopus* embryo" J. Physiol. (Lond.)  
23 489: 489-510). To increase the stability of the  
24 recordings, the head and trunk skin of the embryo  
25 (which is ciliated and thus causes strong water  
26 currents in the bath) was completely removed. The  
27 embryos were bathed in a physiological saline that  
28 contained 115mM NaCl, 3mM KCl, 2mM CaCl<sub>2</sub>, 1mM MgCl<sub>2</sub>, 1mM  
29 NaP<sub>i</sub>, 2.4mM NaHCO<sub>3</sub>, 10mM HEPES, Ph 7.4. The muscles  
30 overlying the spinal cord were removed and the animal  
31 then immobilized in a small recording chamber (0.5ml  
32 volume). Extracellular ventral root recordings were  
33 made to allow swimming activity to be monitored. The  
34 adenosine-sensing probe was carefully aligned with -  
35 and gently pressed onto - the lateral side of the  
36 spinal cord. There was no fluid flow within the

1 recording chamber and the chamber and probe were  
2 carefully shielded from external air currents. Once a  
3 stable background signal had been obtained from the  
4 probe, swimming was evoked by brief (0.5ms) electrical  
5 stimuli to the tail skin of the embryo. The results  
6 are given in Figure 2.

7  
8 **Example 3**

9  
10 **Adenosine biosensor probes**

11 Single and dual-barrelled biosensor probes were  
12 obtained from Sycopel International. Each barrel  
13 consisted of a 230µm diameter semipermeable glass  
14 membrane, a Pt working electrode, an Ag counter  
15 electrode and an Ag-AgCl reference electrode. They  
16 were also fabricated with a 30° bend that allowed the  
17 probe to be placed parallel to the embryo spinal cord  
18 (see Example 4 below). The dual-barrelled probes could  
19 be used as a quasi-differential device, in that enzymes  
20 were loaded into only one barrel and the difference  
21 signal between the two barrels was measured. In this  
22 case the reference and counter electrodes of one barrel  
23 were connected to the equivalent electrodes in the  
24 other barrel.

25  
26 0.05U of adenosine deaminase, 0.1U nucleoside  
27 phosphorylase (both from calf spleen, SIGMA) and 5U of  
28 xanthine oxidase (bacterial, SIGMA) were dissolved in  
29 40µl of saline (115mM NaCl, 1mM NaP<sub>i</sub>, 10mM HEPES, Ph  
30 7.4). 10µl of the enzyme mixture was then loaded, at a  
31 rate of 30µl/hour, into the probe (one barrel for the  
32 dual probes). The probes were controlled by a  
33 potentiostat (Biosensor Driver, Sycopel International;  
34 one for each barrel for the dual probes) that held the  
35 working electrode at +650mV to detect H<sub>2</sub>O<sub>2</sub>.

36

1 For the dual probes the output of the two controlling  
2 biosensors was fed into simple differential amplifier  
3 to provide a signal that was proportional to the  
4 difference between the two probes.

5

#### 6 *In vitro* measurements

7 Calibration and testing of the probe took place in a  
8 vessel (7ml volume). The probe was immersed in saline  
9 that was constantly stirred. To ensure maximum  
10 stability of measurement, care was taken to shield the  
11 vessel and probe from drafts. The adenosine  
12 concentration in the vessel was changed by adding  
13 concentrated aliquots to raise the overall  
14 concentration to known levels. Successive amounts of  
15 adenosine were added to give a calibration curve (Fig.  
16 4). Other agents (eg coformycin and inosine were added  
17 in this way too).

18

19 When loaded into a semipermeable glass microprobe, the  
20 three enzymes completed a biosensor (Fig. 3) that was  
21 very sensitive to adenosine and showed linear responses  
22 from 10nM upwards (Fig. 4a,c,d). In a volume of only a  
23 few hundred  $\mu$ l, this is equivalent to a lower limit of  
24 detection for adenosine of a few pmol. With complete  
25 efficiency in the enzyme cascade, the response to a  
26 given dose of adenosine would be identical to that  
27 resulting from the same dose of inosine. It was found,  
28 by comparing the responses to adenosine and inosine,  
29 that the efficiency was around 80% (Fig. 4b). The  
30 initial enzyme, adenosine deaminase, can be  
31 specifically blocked by coformycin (see Agarwal et al  
32 (1978) Methods in Enzymology 51:502-507). Therefore  
33 50-500nM coformycin was added to the bathing medium.  
34 This blocked the response to adenosine but crucially  
35 had no effect on the response to inosine (Fig. 4b).  
36 Coformycin can therefore be used to block only the

1 first step of the cascade and demonstrate that any  
2 responses rely specifically on the activity of  
3 adenosine deaminase.

4  
5 **Example 4**

6  
7 The biosensor was next used to monitor the production  
8 of adenosine during locomotor activity in the *Xenopus*  
9 embryo spinal cord. ATP and adenosine have important  
10 actions function on the spinal circuitry (see Dale et  
11 al, (1996) Nature 383:259-263) and the changing balance  
12 between these two modulators mediates the run-down and  
13 spontaneous termination of locomotor activity (see Dale  
14 et al (1996) supra). This proposed control system  
15 relies on adenosine being produced with a delay from  
16 synaptically released ATP so that its build-up  
17 throughout motor activity is slow. However direct  
18 evidence for the production of adenosine is lacking; it  
19 remains unclear whether it is produced from the  
20 extracellular breakdown of ATP or is released  
21 synaptically; and no information is available about the  
22 time course of its production.

23  
24 **Measurements of adenosine release from embryo spinal**  
25 **cord**

26  
27 Stage 37/38 *Xenopus* embryos were prepared for recording  
28 by means of well established techniques (Kahn et al  
29 (1982) Journal of Experimental Biology 99:185-196). In  
30 brief, in accordance with the UK Animals (Scientific  
31 Procedures) Act (1986) embryos were anaesthetized in  
32 MS222 and the dorsal fin slit. They were then treated  
33 with  $\alpha$ -bungarotoxin (0.077mg/ml) until they were  
34 immobilised. The trunk skin was then removed and the  
35 muscles overlying one side of the spinal cord from the  
36 hindbrain to the obex were removed to expose the spinal

1 cord. The animal was pinned in a small chamber (0.5ml  
2 volume) so that the lateral side of the exposed cord  
3 was uppermost. Ventral root recordings were made from  
4 the intermyotome clefts and the biosensor probe was  
5 laid along the length of the exposed cord. For dual  
6 probes the barrel with enzymes was in contact with the  
7 cord, while the reference barrel was necessarily  
8 further away (due to the size of the probe relative to  
9 the spinal cord, Fig. 3). Thus the dual probe  
10 recordings were not true differential recordings.  
11 Nevertheless the difference signal was more stable and  
12 less prone to drift and environmental disturbance. The  
13 ventral root recording and output from the biosensor  
14 drivers was plotted on a thermal array recorder  
15 (Graftek). Unlike the *in vitro* measurements, the fluid  
16 in the recording chamber was kept stationary except  
17 during solution changes. The saline for physiological  
18 recordings contained 115mM NaCl, 2.4mM NaHCO<sub>3</sub>, 3mM KCl,  
19 2mM CaCl<sub>2</sub>, 1mM MgCl<sub>2</sub>, 1 or 2mM NaP<sub>i</sub>, 10mM HEPES, pH 7.4.  
20  
21 When the probe was aligned with the ventral portion of  
22 the spinal cord clear responses occurred during motor  
23 activity (Fig. 5). The ventral cord also contains the  
24 densest staining for 5'-nucleotidase activity. The  
25 probe current slowly rose during swimming, and then  
26 after the activity had ceased gradually fell back to  
27 baseline over a period of several minutes (Table 1).  
28 This current was due to release of adenosine from the  
29 spinal cord, since block of adenosine deaminase by 50nM  
30 coformycin greatly reduced the signal from the probe  
31 (n=6). The signals recorded from the probe were  
32 variable depending upon the placement of the probe  
33 relative to the spinal cord. They corresponded to  
34 increases in adenosine concentration ranging from 10nM  
35 to 100nM with a mean change of 58nM (n=13, Fig. 5a,  
36 Table 1). In 4 additional experiments the change in



1 adenosine levels was much larger and ranged from 150 to  
2 nearly 650nM (mean change 377nM, Fig. 5b, Table 1).  
3 These large signals could also be blocked with  
4 coformycin (Fig. 5b) and were presumably recorded  
5 because the probe was fortuitously placed very close to  
6 the source of adenosine production. In these 4 cases,  
7 the levels of adenosine continued to rise for 15-72s  
8 beyond the end of the episode before falling back to  
9 baseline (Table 1). This behaviour may be expected if  
10 adenosine is produced from a pool of AMP that  
11 accumulates in the extracellular space and persists  
12 after neural activity has finished.  
13

Table 1

Type	Change in [Adenosine] (Nm)	Half decay time (sec)	Delay to peak (sec) (peak-end)	Ratio (peak/end)
Small (n=13)	58±6	90±19.3	6.5±4.8	1.1±0.1
Large (n=4)	377±106	104±16.8	49±12.3	2.0±0.4

18 Magnitude and time course of adenosine-production  
19 during swimming. The data are divided into two groups  
20 dependent upon size of adenosine response (see text).  
21 The "half decay time" is the time for the adenosine  
22 level to fall to half its peak value; the "delay to  
23 peak" refers to the delay between the end of a swimming  
24 episode and the peak of the adenosine response; and the  
25 "ratio" is the peak concentration of adenosine divided  
26 by that achieved at the end of the episode of swimming.  
27 All values expressed as a mean ± sem. The n numbers  
28 refer to the number of preparations.  
29  
30

### 31 Example 5

32 To test whether adenosine uptake systems could play a  
33 role in limiting the rise of adenosine during locomotor

1 activity, the effects of NBTG a blocker of adenosine  
2 uptake, were studied. At  $1\mu\text{M}$ , NBTG had two effects  
3 (Fig. 6): it greatly enhanced the magnitude (means  $60$   
4  $\pm 9$  and  $175 \pm 44$   $\text{Nm}$  in control and NBTG respectively,  
5  $n=5$ ) and rate of the rise in adenosine concentration  
6 (means  $37 \pm 7$  and  $101 \pm 34$   $\text{Nm}\cdot\text{min}^{-1}$  in control and NBTG  
7 respectively,  $n=5$ ); and it slowed the recovery after  
8 the cessation of motor activity (in 3 of 5 preparations  
9 the probe signal did not decay to half peak within 5  
10 minutes). This result suggests that adenosine uptake  
11 plays an important role in slowing and limiting the  
12 rise in adenosine concentrations during activity.

13  
14 That levels of adenosine can continue to rise even  
15 after locomotor has ceased, effectively rules out the  
16 possibility that adenosine is released from neurons as  
17 a transmitter. Instead, it strongly suggests that it  
18 is produced from the breakdown of synaptically released  
19 ATP via an extracellular intermediate. The possible  
20 time course of ATP catabolism was analysed by modifying  
21 a model for ectonucleotidase action that was originally  
22 proposed for endothelial cells (see Gordon et al.  
23 (1986) Journal of Biological Chemistry 261: 15496-  
24 15504). This earlier work used Michaelis-Menten  
25 kinetics to describe the actions of each enzyme, and  
26 incorporated feed-forward inhibition by ADP of the  
27 conversion of AMP to adenosine as described below.

#### 28 29 Simulation of breakdown of ATP

30 The methods and equations of Slakey (1986) Journal of  
31 Biological Chemistry 261: 15505-15507 were adapted. In  
32 brief, the breakdown of ATP was considered as 4 coupled  
33 irreversible reactions (through ADP, AMP and finally  
34 adenosine). The velocity of each reaction (without  
35 feed-forward inhibition) was described by the following  
36 equation:

17

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

The four coupled reactions were:

$$\frac{d[ATP]}{dt} = -v_{ATP} + k_R \quad (2)$$

$$\frac{d[ADP]}{dt} = v_{ATP} - v_{ADP} \quad (3)$$

$$\frac{d[AMP]}{dt} = v_{ADP} - v_{AMP} \quad (4)$$

$$\frac{d[ADO]}{dt} = v_{AMP} - v_U \quad (5)$$

where  $k_R$  is the rate of release of ATP (and was set to 3 during swimming and 0 at other times);  $v_{ATP}$ ,  $v_{ADP}$  and  $v_{AMP}$  are the velocities of breakdown of ATP, ADP and AMP and  $v_U$  is the velocity of adenosine-uptake. The velocities  $v_{ATP}$ ,  $v_{ADP}$  and  $v_U$  were calculated according to equation (1). However, to incorporate competitive inhibition by ADP of the breakdown of AMP,  $v_{AMP}$  was described by the following equation:

$$v_{AMP} = \frac{V_{max}[S]}{K_m(1 + \frac{[ADP]}{K_i}) + [S]} \quad (6)$$

where  $K_i$  is the equilibrium constant of inhibition. The parameters used are taken from Slakey et al and are summarized in Table 1. The four differential equations (2-5) were integrated numerically using a Runge-Kutta fourth order algorithm with adaptive step size control (see Press et al (1988) Numerical recipes in C. The art of Scientific computing Cambridge University Press). Simulations were run on a Sun Ultra 170E.

Without feed-forward inhibition of the breakdown of AMP, the peak of adenosine concentration occurred close to the end of the episode of activity (Fig. 6b).

1     However when feed-forward inhibition was introduced,  
2     AMP accumulated during the activity and the build-up of  
3     adenosine was slowed and its peak concentration was  
4     delayed until well after the end of activity (Fig. 6c,  
5     compare to Fig. 4b). These new observations directly  
6     demonstrate that adenosine is produced from ATP in the  
7     extracellular space and strongly support the existence  
8     of feed-forward inhibition to slow the build-up of  
9     adenosine. This suggests, in turn, that the run-down  
10    of motor activity depends very strongly on the nature  
11    of the feed-forward inhibition of the 5'-nucleotidase.

12  
13    A period of relative refractoriness for motor activity  
14    follows swimming episodes in the *Xenopus* embryo. To  
15    reliably elicit episodes of consistent length, a gap of  
16    at least 3 minutes must elapse between the end of one  
17    episode and the onset of the next (see Wall and Dale  
18    (1995) *Journal of Physiology* 487: 557-572). As this  
19    period correlates well with the elevated levels of  
20    adenosine that follow an episode of swimming, the  
21    persistence of adenosine in the extracellular space may  
22    contribute to the transient refractoriness of spinal  
23    circuits following motor activity.

24  
25    This new method could be adapted to allow real-time  
26    measurement of adenosine production both in brain  
27    slices and freely behaving animals. In both cases the  
28    ability to perform rapid determination of adenosine  
29    levels and specifically relate any changes to neural  
30    activity should greatly enhance our understanding of  
31    the functional roles of adenosine. This technique  
32    could be used in a device capable of the rapid  
33    determination of adenosine in human blood and CSF which  
34    may be of value in the diagnosis and treatment of  
35    disorders of the heart and circulation, asthma and  
36    neurological deficits resulting from faulty regulation

1 of adenosine.

2

3 Table 2

4	ATP	ADP	AMP	Adenosine uptake
5 $v_{\max}$	22	3.2	3.0	1
6 $K_m$	333	95	9.4	10
7 $K_i$	-	3.3	-	-

8

9 Kinetic parameters used in model for simulation of  
10 breakdown of ATP. Units for  $v_{\max}$  are arbitrary while  
11 those for  $K_m$  and  $K_i$  are in  $\mu\text{M}$ .

12

13 FIGURE LEGENDS

14

15 Figure 1 - Detection of adenosine *in vitro* by enzyme  
16 microprobe

17 A A dual probe was run in quasi-differential mode

18 with enzymes present in only one barrel. The

19 difference signal between the two probes is

20 plotted against time as successive additions to

21 adenosine raise the bath concentration of

22 adenosine to 10, 20, 40, 80 and 160nM.

23

24 B Plot of the peak current response versus

25 concentration of added adenosine. The response is

26 linear and has a slope of 3.6 nM/pA.

27

28 Figure 2 - Detection of adenosine-release during

29 swimming activity in a *Xenopus* embryo

30 A Top trace (probe) is the signal from the dual

31 probe in differential mode. The bottom trace

32 (v.r.) is the ventral root activity recorded from

33 a paralysed embryo. Although the embryo is

34 paralysed it can still produce the appropriate

35 neural commands to control swimming and these are

36 monitored by the ventral root electrode. Swimming

1 activity was elicited by an electrical stimulus to  
2 the skin at \*. The episode lasts nearly 3 minutes  
3 before spontaneously stopping. During swimming  
4 the signal from the adenosine probe gradually  
5 rises. Once the episode of swimming finishes, the  
6 signal from the probe falls back to baseline.

7  
8 B The specific signal related to adenosine can be  
9 blocked by EHNA, an inhibitor of adenosine  
10 deaminase. In the same preparation as (A) EHNA  
11 was added to the bath and swimming evoked. Only a  
12 much smaller, non-specific signal is seen.

13  
14 Figure 3. The principle behind the enzymatic-  
15 electrochemical detection of adenosine.

16 Schematic of a dual biosensor probe 20 lying parallel  
17 to the spinal cord 18 (drawn roughly to scale, Top).  
18 Inside one barrel 16, the three enzymes of the cascade  
19 are present. Inside the other barrel 14, no enzymes  
20 are present. Adenosine diffuses from myomers 22  
21 through the semipermeable glass membrane 10 and is  
22 successively metabolized to uric acid with the  
23 liberation of  $H_2O_2$  which then donates electrons to the  
24 Pt working electrode 12 at which is applied a voltage  
25 of +650mV. The current detected is thus proportional  
26 to the amount of adenosine present.

27  
28 Figure 4. *In vitro* calibration and characterization of  
29 the adenosine biosensor.

30 (a) Successive amounts of adenosine were added to the  
31 bath at each arrow to raise the concentration of  
32 adenosine in the bath by the amount indicated under  
33 each arrow. The change in probe current resulting from  
34 each application of adenosine is plotted in (c). This  
35 shows that probe responds in a linear fashion.

36

1 (b) In the same experiment 80nM inosine was added  
2 (immediately after the 80nM adenosine). The response  
3 to inosine (substrate for the second enzyme in the  
4 cascade) was about 25% bigger than the response to the  
5 same amount of adenosine, indicating some loss of  
6 efficiency in the probe. 500nM coformycin, a specific  
7 blocker of adenosine deaminase was added. This rapidly  
8 reduced the probe current (due to the continued  
9 presence of adenosine in the bath) and greatly  
10 attenuated the response to subsequent addition of  
11 adenosine. However the response to inosine was  
12 unaffected. Thus coformycin only disables the first  
13 part of the cascade but leaves the rest intact making  
14 it a good test for the specificity of the device.

15  
16 (d) After the coformycin had been washed out, the  
17 sensitivity of the probe to adenosine recovered  
18 (although it was still slightly lower than in c). This  
19 calibration shows that the response to adenosine was  
20 linear from 10nM to 2 $\mu$ M.

21  
22 Figure 5: The biosensor probe can detect adenosine  
23 released from the spinal cord during fictive  
24 locomotion.

25 (a) Production of adenosine during two consecutive  
26 episodes of swimming monitored by a ventral root  
27 recording (v.r.). Note the slow rise in the probe  
28 current and the slow decay after the end of swimming  
29 episode. The increases in probe current are equivalent  
30 to a change in adenosine concentration of about 60nM.  
31 The record at the right shows that application of 50nM  
32 coformycin blocks most of the probe current indicating  
33 that the signal is largely due to the release of  
34 adenosine.

35  
36 (b) Example (from another preparation) where favourable

1 placement of the probe relative to the spinal cord  
2 resulted in a massive signal equivalent to a change in  
3 adenosine concentration of about 370nM. In this case  
4 there is a fast component to the probe current (arrow)  
5 seen at the beginning of the swimming activity. Note  
6 that the probe signal continues to rise for about 50s  
7 after the end of the swimming episode. Application of  
8 50nM coformycin blocked the probe current, but left a  
9 small fast component. The large slowly developing  
10 component of the probe current was thus specifically  
11 due to the release of adenosine.

12

13 **Figure 6. Blockers of adenosine uptake greatly**  
14 **enhanced the release of adenosine from the spinal cord.**  
15 (a) In the control (left) the probe current involved  
16 both fast (arrow) and slow components, the slow  
17 component being equivalent to a rise in adenosine  
18 concentration of around 64nM. After application of 1μM  
19 NBTG (right) to block adenosine-uptake, the fast  
20 component (arrow) was unchanged, but the slow component  
21 was greatly increased in amplitude and rate of rise  
22 (equivalent to a change of about 150nM).

23

24 (b) Simulation of the breakdown of ATP without feed-  
25 forward inhibition by ADP. The peak of adenosine  
26 concentration is only lightly delayed relative to the  
27 end of a swimming episode (shown by bar).

28

29 (c) When feed-forward inhibition is incorporated, AMP  
30 accumulates and the peak of adenosine concentration  
31 occurs well after the cessation of activity. The trace  
32 for ATP is unmarked in both panels. Both the  
33 concentration and time scales are in arbitrary units.

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# CLAIMS

1. A biosensor to detect adenosine which comprises the enzymes adenosine deaminase, nucleoside phosphorylase and xanthine oxidase, or functional equivalents thereof, immobilized on a support and means to detect hydrogen peroxide.
2. A biosensor as claimed in Claim 1, wherein said detection means comprises an electrolytic probe.
3. A biosensor as claimed in Claim 1 or 2, wherein said enzymes are in an aqueous environment.
4. A biosensor as claimed in any of Claims 1 to 3, wherein said enzymes are immobilised on a semi-porous membrane.
5. A biosensor as claimed in Claim 4, wherein said semi-porous membrane is a semi-permeable glass membrane.
6. A biosensor as claimed in any of Claims 1 to 5, which comprises a single or a dual-barrelled probe, said probe consisting of a glass membrane, an electrolytic cell which comprises a working electrode, a counter electrode and a reference electrode and wherein the said enzymes are immobilized onto said glass membrane.
7. A biosensor as claimed in Claim 6, wherein

1 said working electrode is platinum, said  
2 counter electrode is silver and said  
3 reference electrode is a silver/silver  
4 chloride type electrode.  
5

6 8. A biosensor as claimed in either of Claims 6  
7 and 7, wherein said glass membrane is  
8 cylindrical in shape and has a diameter  
9 ranging from about 200 and 300 $\mu$ m.  
10

11 9. A biosensor as claimed in any of Claims 6 to  
12 8, wherein said probe is a dual-barrelled  
13 probe which is used as a quasi-differential  
14 device and wherein said enzymes are  
15 immobilized on only one of the barrels and  
16 the difference signal between the two barrels  
17 measured.  
18

19 10. A biosensor as claimed in any of Claims 1 to  
20 9, wherein the ratio of adenosine deaminase:  
21 nucleoside phosphorylase is in a range from  
22 1:100 to 1:1, especially 1:10 to 1:1, and  
23 wherein ratios of nucleoside phosphorylase:  
24 xanthine oxidase is in a range from 1:100 to  
25 1:10, especially 1:50 to 1:10.  
26

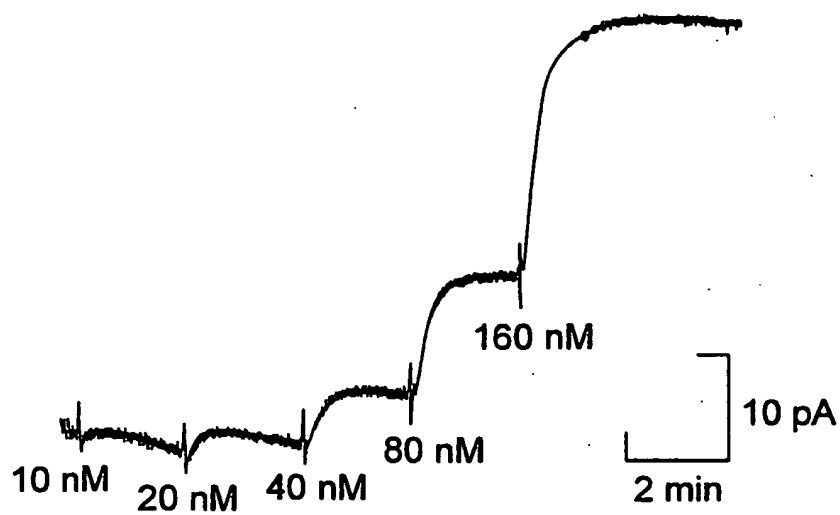
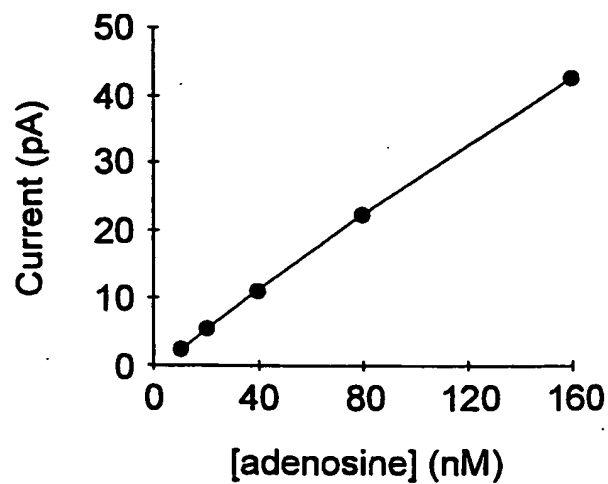
27 11. A biosensor as claimed in Claim 10, wherein  
28 the ratio of adenosine deaminase:nucleoside  
29 phosphorylase:xanthine oxidase is in a range  
30 from 1:1:50 to 1:5:200.  
31

32 12. A biosensor as claimed in Claim 11, wherein  
33 the ratio of adenosine deaminase:nucleoside  
34 phosphorylase:xanthine oxidase is  
35 approximately 1:2:100.  
36

- 1     13. A method of detecting adenosine in a sample,  
2       said method comprising exposing the sample to  
3       the enzymes adenosine deaminase, nucleoside  
4       phosphorylase and xanthine oxidase, or  
5       functional equivalents thereof, such that the  
6       enzymes can act sequentially on the sample,  
7       and measuring the production of hydrogen  
8       peroxide there from.  
9
- 10    14. A method of monitoring the amount of  
11       adenosine in an patient which comprises  
12       repeatedly measuring the amount of hydrogen  
13       peroxide in a patient over time according to  
14       the method as claimed in Claim 13.  
15
- 16    15. A method of diagnosis and treatment of  
17       pathological conditions that result from  
18       faulty regulation of adenosine, said method  
19       comprising detecting the levels of adenosine  
20       in a patient by exposing at least one sample  
21       of said patient to the enzymes adenosine  
22       deaminase, nucleoside phosphorylase and  
23       xanthine oxidase, or functional equivalents  
24       thereof; such that the enzymes can act  
25       sequentially on the sample; and measuring the  
26       production of hydrogen peroxide there from.  
27
- 28    16. A method of monitoring drug requirements in a  
29       patient, wherein said drug affects the *in*  
30       vivo levels of free adenosine in a body fluid  
31       or an organ of said patient, said method  
32       comprising detecting the level of adenosine  
33       as described in Claim 13.  
34
- 35    17. A method of monitoring adenosine levels in a  
36       patient before, during and/or after surgery,

- 1 wherein the adenosine levels are detected
- 2 according to the method described in Claim
- 3 14.
- 4

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*Fig. 1A**Fig. 1B*

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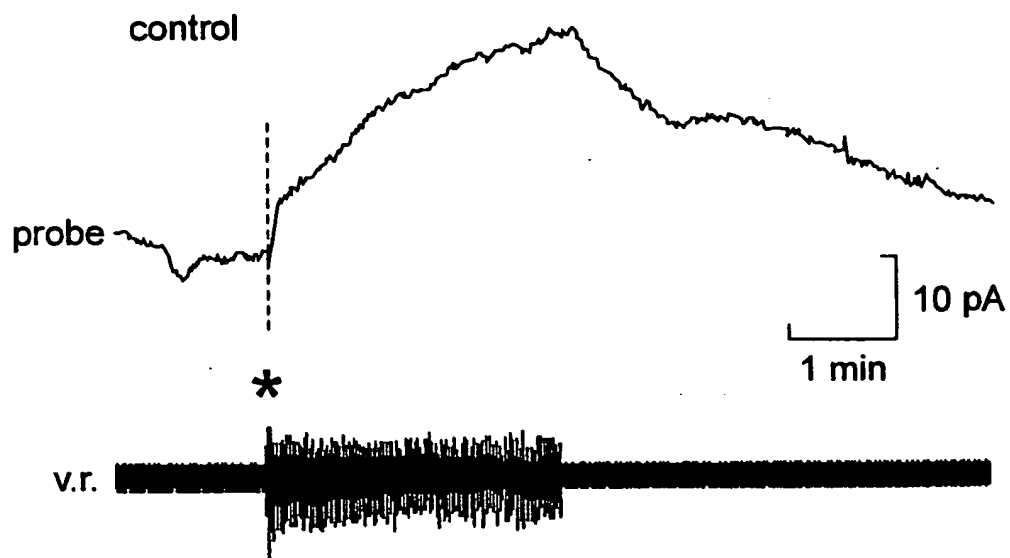


Fig. 2A

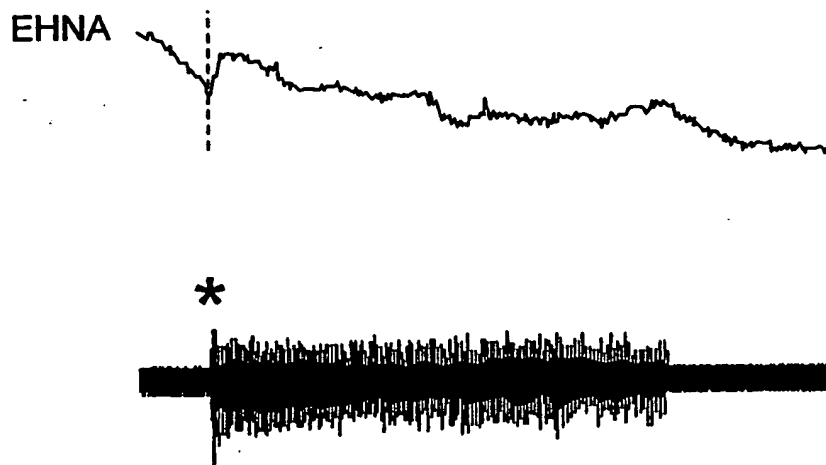


Fig. 2B

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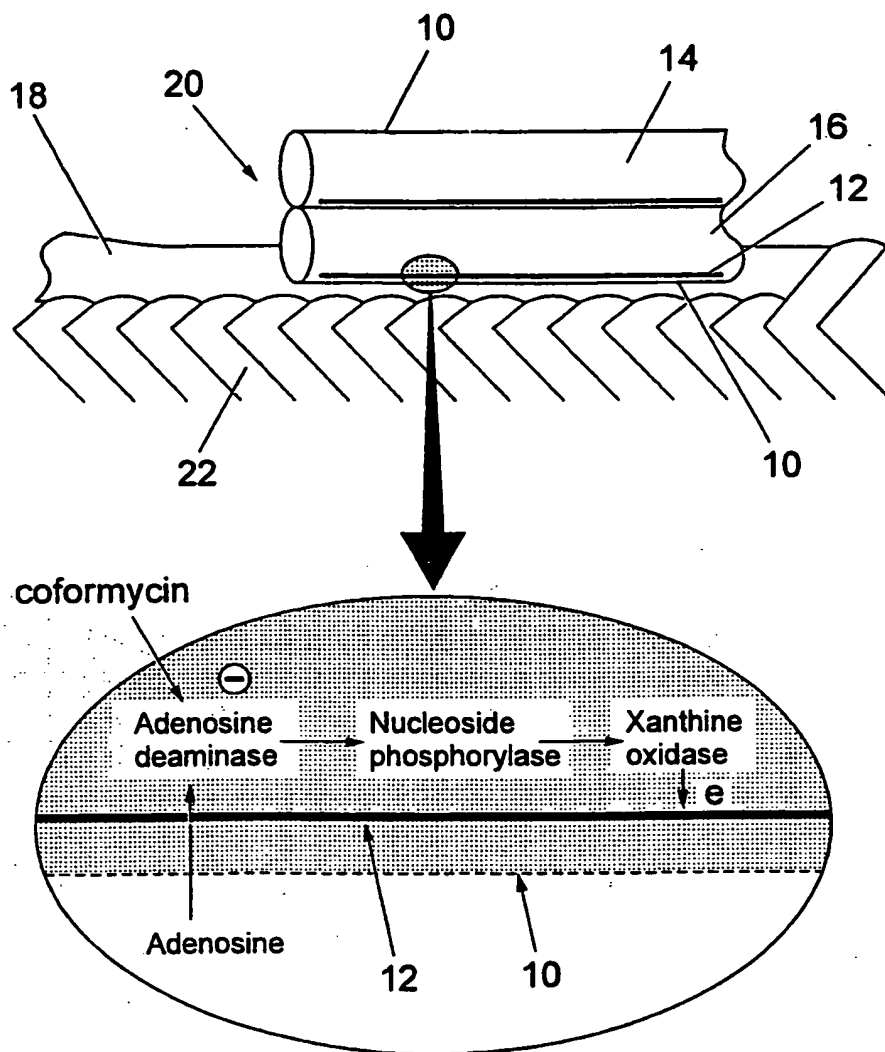


Fig. 3

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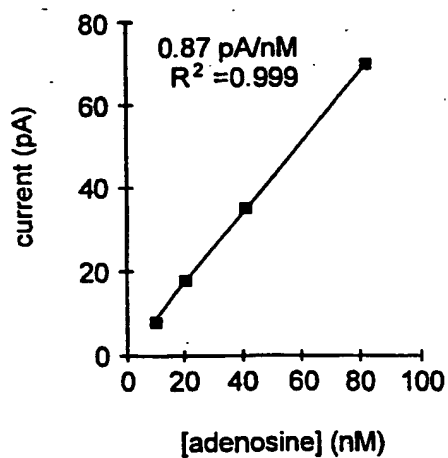
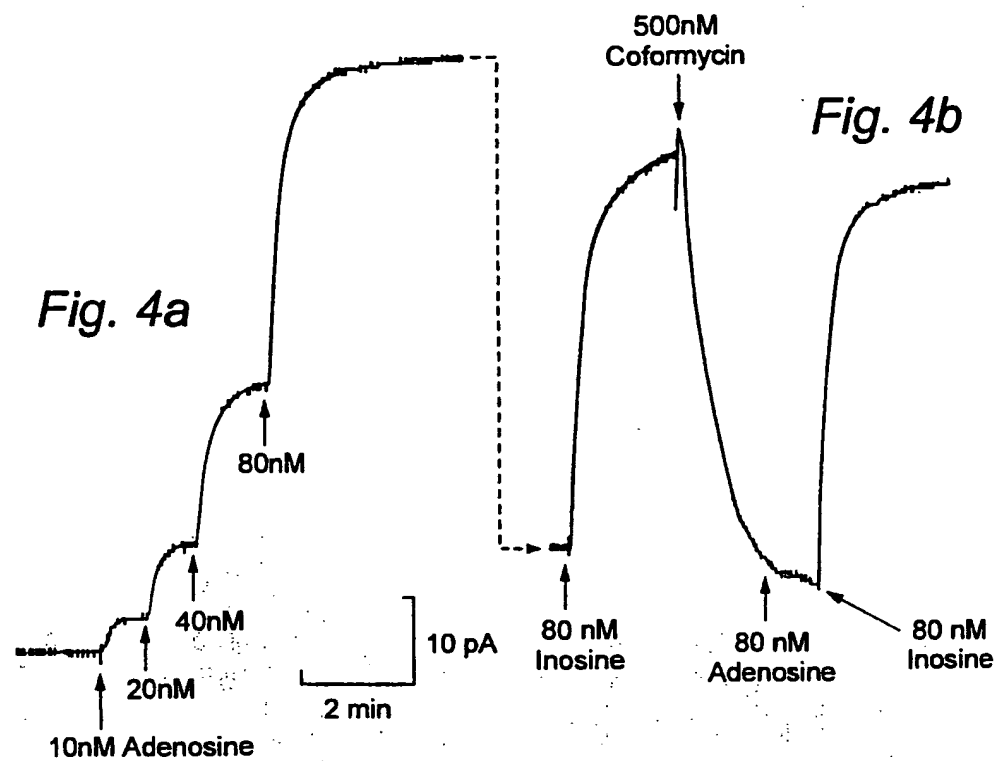
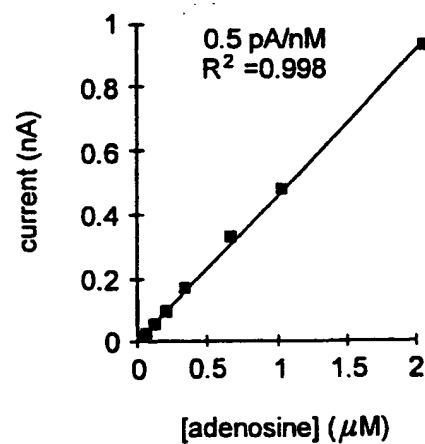
**Fig. 4c****Fig. 4d**





Fig. 5a

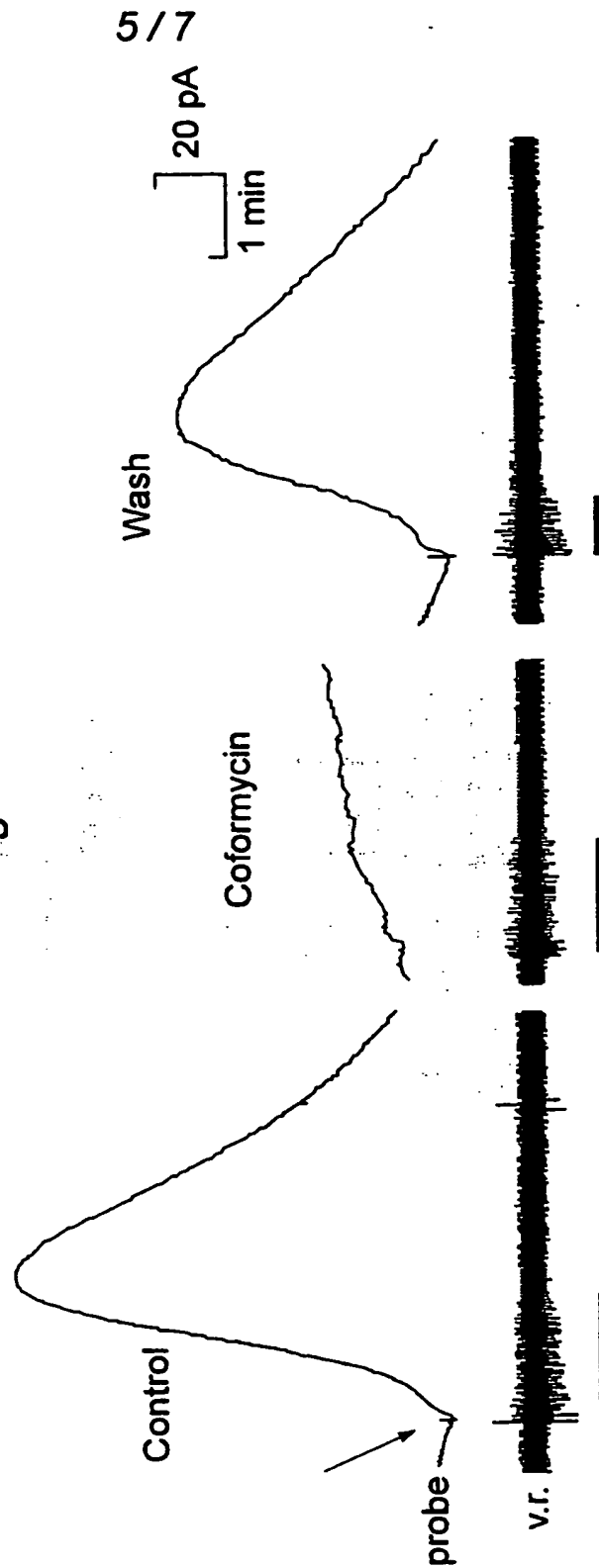


Fig. 5b

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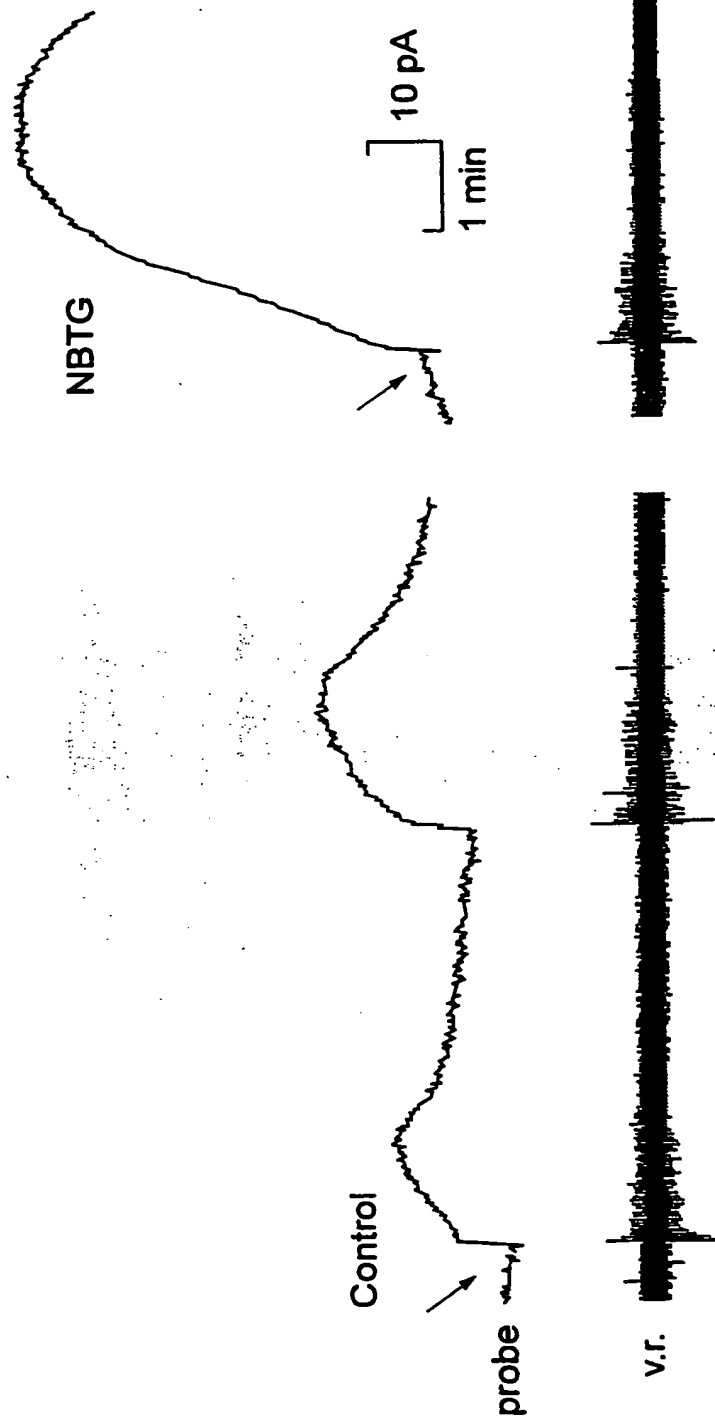


Fig. 6a

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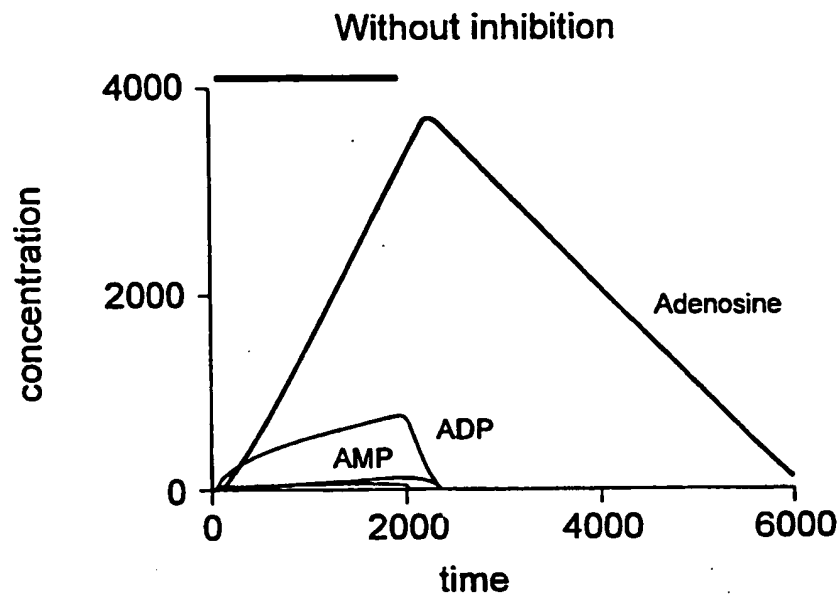


Fig. 6b

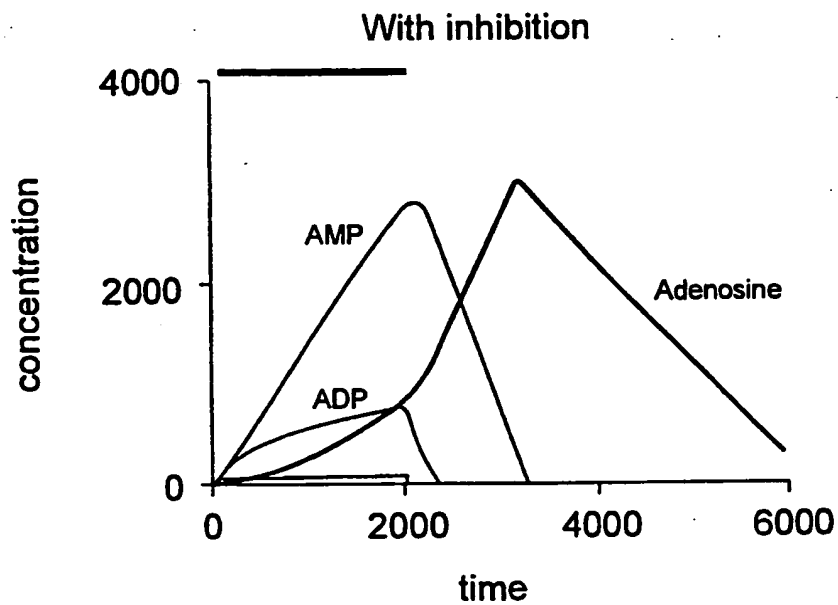


Fig. 6c

# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/GB 98/02239

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/00 G01N27/327 C12Q1/34 C12Q1/48 C12Q1/26

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 288 613 A (LUONG JOHN H T ET AL) 22 February 1994 see column 3, line 56 - column 4, line 60; claim 10 see abstract	1-13
X	PATENT ABSTRACTS OF JAPAN vol. 096, no. 012, 26 December 1996 & JP 08 205891 A (NEW JAPAN RADIO CO LTD), 13 August 1996 see abstract	1-5



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

22 October 1998

Date of mailing of the international search report

05/11/1998

Name and mailing address of the ISA

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Moreno, C

# INTERNATIONAL SEARCH REPORT

Int. Patent Application No.

PCT/GB 98/02239

## C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI Section Ch, Week 8808 Derwent Publications Ltd., London, GB; Class B04, AN 88-054472 XP002081753 &amp; JP 63 011848 A (ORIENTAL ELECTRIC CO LTD), 19 January 1988 see abstract</p>	1-5
X	<p>Y. HAYASHI ET AL.: "Flow-injection determination of adenosine and inosine in blood plasma with immobilized enzyme columns connected in series and fluorimetric detection." ANALYTICA CHIMICA ACTA, vol. 186, 1986, pages 131-137, XP002081750 see the whole document</p>	1,13
P,X	<p>C. D. T. BRATTEN ET AL.: "Single-cell measurements of purine release using a micromachined electroanalytical sensor." ANALYTICAL CHEMISTRY, vol. 70, no. 6, 15 March 1998, pages 1164-1170, XP002081751 COLUMBUS US see abstract</p>	1,13
A	<p>US 5 534 504 A (SOLLEVI ALF) 9 July 1996 see the whole document</p>	15
A	<p>H. KATHER ET AL: "Chemiluminescent determination of adenosine, inosine, and hypoxanthine/xanthine." ANALYTICAL BIOCHEMISTRY, vol. 163, no. 1, 1987, pages 45-51, XP002081752 see the whole document</p>	1,13

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 98/02239

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 14-17  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 14-17  
are directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/02239

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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